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Research review paper

Pluripotent stem cell-derived hepatocyte-like cells

R.E. Schwartz [a,c,e], H.E. Fleming [a,c,d], S.R. Khetani [f], S.N. Bhatia [a,b,c,d,e,*]

[a] Harvard-MIT Division of Health Sciences and Technology, Institute for Medical Engineering and Science, Massachusetts Institute of Technology, Cambridge, MA, USA
[b] Department of Electrical Engineering and Computer Science, Massachusetts Institute of Technology, Cambridge, MA, USA
[c] David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA, USA
[d] Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, MA, USA
[e] Department of Medicine, Brigham and Women's Hospital, USA
[f] Mechanical and Biomedical Engineering, Colorado State University, Fort Collins, CO, USA

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ABSTRACT

Liver disease is an important clinical problem, impacting over 30 million Americans and over 600 million people worldwide. It is the 12th leading cause of death in the United States and the 16th worldwide. Due to a paucity of donor organs, several thousand Americans die yearly while waiting for liver transplantation. Unfortunately, alternative tissue sources such as fetal hepatocytes and hepatic cell lines are unreliable, difficult to reproduce, and do not fully recapitulate hepatocyte phenotype and functions. As a consequence, alternative cell sources that do not have these limitations have been sought. Human embryonic stem (hES) cell- and induced pluripotent stem (iPS) cell-derived hepatocyte-like cells may enable cell based therapeutics, the study of the mechanisms of human disease and human development, and provide a platform for screening the efficacy and toxicity of pharmaceuticals. iPS cells can be differentiated in a step-wise fashion with high efficiency and reproducibility into hepatocyte-like cells that exhibit morphologic and phenotypic characteristics of hepatocytes. In addition, iPS-derived hepatocyte-like cells (iHLCs) possess some functional hepatic activity as they secrete urea, alpha-1-antitrypsin, and albumin. However, the combined phenotypic and functional traits exhibited by iHLCs resemble a relatively immature hepatic phenotype that more closely resembles that of fetal hepatocytes rather than adult hepatocytes. Specifically, iHLCs express fetal markers such as alpha-fetoprotein and lack key mature hepatocyte functions, as reflected by drastically reduced activity (~0.1%) of important detoxification enzymes (i.e. CYP2A6, CYP3A4). These key differences between iHLCs and primary adult human hepatocytes have limited the use of stem cells as a renewable source of functional adult hepatocytes for in vitro and in vivo applications. Unfortunately, the developmental pathways that control hepatocyte maturation from a fetal into an adult hepatocyte are poorly understood, which has hampered the field in its efforts to induce further maturation of iPS-derived hepatic lineage cells. This review analyzes recent developments in the derivation of hepatocyte-like cells, and proposes important points to consider and assays to perform during their characterization. In the future, we envision that iHLCs will be used as in vitro models of human disease, and in the longer term, provide an alternative cell source for drug testing and clinical therapy.

Contents

* Corresponding author at: Harvard-MIT Division of Health Sciences and Technology, Institute for Medical Engineering and Science, Massachusetts Institute of Technology, Cambridge, MA, USA.
E-mail address: sbhatia@mit.edu (S.N. Bhatia).

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1. Introduction

Chronic liver disease is a significant cause of morbidity and mortality, impacting over 600 million people worldwide (Gonzalez and Keeffe, 2011). As a result, the number of people living with end stage liver disease is increasing, and over 1 million people die each year from acute and chronic liver disease across the globe (Gonzalez and Keeffe, 2011). Liver transplantation is currently the only definitive and curative treatment for acute and chronic liver failure (Starzl and Fung, 2010). First accomplished in 1967 by Thomas Starzl, liver transplantation has been an unquestioned clinical success; however, the demand for liver transplantation has significantly outstripped the supply of donor organs (Perera et al., 2009; Starzl and Fung, 2010; Starzl et al., 1968). As a consequence, multiple attempts to expand the availability of donor organs have been employed: opt-out organ donation programs, the use of suboptimal donor organs (deceased cardiac donors or steatotic (fatty) livers), split donor transplantation, and living donor liver transplantation (Perera et al., 2009).

The search for alternatives to whole organ transplantation has been focused on expanding the availability of replacement liver tissue, such as developing cell-based therapies that include hepatocyte transplantation, engineered hepatic tissue constructs, and the bioartificial liver (Chen et al., 2011; Dhawan et al., 2010; Fox and Roy-Chowdhury, 2004; Fox et al., 1998; Nyberg et al., 1993). In particular, hepatocyte transplantation has been performed clinically for more than 15 years, primarily in the setting of acute liver failure and inherited liver metabolic disorders. A general problem facing hepatocyte transplantation is the limited repopulation capacity of engrafted cells, although in the case of some metabolic disorders, replacement of just 2–5% of the liver parenchyma with normal hepatocytes may be sufficient to improve liver function significantly. For example, Fox et al. reported the successful treatment of a 10-year-old with one such metabolic disorder, termed Crigler–Najjar disease, who was experiencing recurrent episodes of brain injury resulting from elevated bilirubin. The patient was shown to respond well to infusion of 7.5×10^9 hepatocytes, based on an improvement in metabolic function and reduced need for phototherapy (Fox et al., 1998). However, hepatocyte transplantation has not been widely adopted, due to a variety of technical reasons including the inability to monitor graft health and frequent signs of rejection (Dhawan et al., 2010). Moreover, these clinical treatments require scarce human liver tissue as a cell source of the transplanted hepatocytes.

Based on the apparent success of hepatocyte transplantation combined with the challenges in sourcing appropriate donor cells, a strong focus has been placed on developing a safe and reliable method to expand the small number of available human hepatocytes. Indeed, the liver has been known for its capacity to regenerate since antiquity, as depicted by the story of Prometheus. Modern studies have shown that in vivo, human hepatocytes are capable of cellular proliferation based on the observed replacement of damaged hepatocytes following injury, or even during the daily turnover of the liver (Michalopoulos, 2007). However, in vitro, researchers have been unable to induce and/or support the cellular proliferation of human hepatocytes; rather, attempts to culture human hepatocytes have led to the loss of differentiated function rather than any increase in cell number (Castell et al., 2006; Kobayashi et al., 2000). Consequently, attempts to expand adult human hepatocytes have historically been unsuccessful as a target approach to achieving cellular therapy of the liver, although alternatives are under active investigation, including our recent screen that identified small molecules that support up to 10-fold expansion of adult human hepatocytes in vitro (Shan et al., 2013). Other approaches include utilizing cell lines derived from hepatocellular carcinoma, or generated through SV40 or Large T antigen transformation, both of which have enabled the expansion and creation of in vitro model systems (Ito et al., 2009). However, these cell lines poorly recapitulate primary hepatocyte functions such as detoxification enzyme activities and thus show poor prediction of clinical outcomes such as drug toxicity(Gerets et al., 2012; Wilkening et al., 2003).

2. Hepatocyte differentiation from pluripotent stem cells

Several alternative sources have been proposed as options to circumvent the limited supply of human hepatocytes, including using human fetal tissue or even xenogeneic material, but both paths have been sidelined due to a variety of ethical, sourcing, and safety issues (Yu et al., 2012). While still prone to some ethical and safety challenges, pluripotent stem cell-based therapies overcome many of the drawbacks that challenge other cell lines and fetal tissue, and thus are considered by many as an ideal alternative source of human hepatocytes (Dalgetty et al., 2009; Espejel et al., 2010). Human pluripotent stem cells include embryonic stem (hES) cells, first isolated from human blastocysts by James Thomson and colleagues (Thomson et al., 1998), as well as the more recently described induced pluripotent stem (iPS) cells first generated by Yamanaka and colleagues following the forced expression of a panel of transcription factors in adult-derived cells (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). These cell lines are defined as pluripotent in that they can self-renew in culture, maintain genetic stability, and differentiate into cell lineages of all three germ layers including endodermal hepatocyte-like cells (HLCs) (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Thomson et al., 1998). Importantly, iPS cells can be derived from adult tissue in a reliable manner and have been shown to differentiate efficiently into hepatocyte-like cells (Si-Tayeb et al., 2010; Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Touboul et al., 2010). However, unlike relatively simple in vitro models designed to reproduce embryonic differentiation, in vivo development advances through a much more complex, structured and highly organized series of patterning and differentiation events in which cell–extracellular matrix and cell–cell interactions are tightly controlled and play an important role (Deutsch et al., 2001; Matsumoto et al., 2001; Wandzioch and Zaret, 2009; Zaret and Grompe, 2008). Consequently, the hepatocyte-like cells generated from pluripotent stem cells in culture exhibit many morphologic and phenotypic characteristics of primary adult human hepatocytes. However, the examination of their functional traits has been more limited, and many signs suggest that only partial differentiation has been attained, as discussed below.

Despite the challenges inherent in performing developmental studies in an in vitro setting, and the roadblocks that remain regarding the current capacity to treat patients with human hepatocytes from any available derivation source, the importance of being able to develop experimental models to study human disease states cannot be overstated. To date, many genome-wide association screens (GWAS) have identified a variety of genetic variants associated with human liver disease (Ott et al., 2011). However, many of these variants represent novel loci whose contribution to liver disease is entirely unknown. Linking GWAS findings to biologic mechanisms has been an ongoing challenge in the genetics community. In most studies, mouse models have been employed; however, the usefulness of mouse models is unclear given its low-throughput nature and the physiologic and metabolic

differences between humans and rodents (Schmouth et al., 2012; Schofield et al., 2012). Similarly, zebrafish models have been employed as a higher-throughput system to examine numerous genetic variants, but given the significant developmental, structural, physiologic, and metabolic differences between species, the value of these models is less clear (Daly, 2012; Lieschke and Currie, 2007). Consequently, cell culture systems have been employed, despite the observation that although no single cell type tested to date fully recapitulates hepatocyte morphology, phenotype, and functions (Deo and MacRae, 2011; Norton et al., 2011; Pattaro et al., 2012). In contrast, iPS cells offer the potential to establish patient-specific cell types such as iPS-derived hepatocyte-like cells (iHLCs), thus facilitating in vitro modeling of rare diseases, and may one day enable personalized medicine (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). When combined with the capacity to engineer genetic changes in established iPS lines (Soldner et al., 2011), patient-specific iPS cells and iHLCs can be utilized to study genetic variants identified in GWAS studies, as well as a host of other monogenic alterations to assess their impact on hepatocyte differentiation, phenotype and functions (Cayo et al., 2012). For example, recent papers have demonstrated that iHLCs can recapitulate the disease phenotype of alpha-1-antitrypsin disease, familial hyperlipidemia, and Wilson's disease, although the capacity to apply this approach to study polygenic disorders remains to be seen (Cayo et al., 2012; Rashid et al., 2010; Zhang et al., 2011). And yet, in order to maximize the potential use of iHLCs either for studies of disease models and treatment, or for eventual cell transplantation therapies, it is essential that efficient and reproducible iHLC differentiation protocols be established.

Significant progress has been made over the past few years in the derivation of iHLCs and consequently, a multitude of differing protocols have been developed (Moore et al., 2008; Sharma et al., 2009; Si-Tayeb et al., 2010; Song et al., 2009; Touboul et al., 2010). Most of these protocols share general themes regarding their approach to differentiation induction, however, specific differences are apparent upon close examination of the individual methodologies. Here, we review some of the most established and cited iHLC methods. We stress the importance of detailed characterization of derived cell types, and that multiple phenotypic and functional readouts are required in this effort. Based on an analysis of the available literature, we provide a summary of assays that can be applied during the analysis of iHLC populations, towards the goal of promoting a unified field and achieving a robust, mature source of liver cells.

2.1. Hepatic differentiation protocols

For decades, researchers have attempted to promote the in vitro differentiation of primitive, and now pluripotent cells towards specific lineages of all three germ layers. A typical approach to designing such protocols has been to mimic the patterns and stages observed during embryologic development, in order to recreate the necessary molecular and cellular cues. In the case of pluripotent cells, most protocols apply either one of several cellular aggregation strategies or promote differentiation in a monolayer culture. Pluripotent stem cells can be aggregated in suspension or using specialized plates, which results in the formation of three-dimensional structures called embryoid bodies (EBs) that may serve to replicate some of the cell–cell and cell–extracellular matrix (ECM) signals that are experienced during development in vivo (Ungrin et al., 2008). By culturing EBs in specific cytokine cocktails or on different ECM – again, designed in an effort to mimic signals observed during embryonic liver development – it is possible to improve the efficiency of iHLC generation (Schwartz et al., 2005). However, all existing protocols still suffer from relatively low differentiation efficiency, and tend to lead to the production of a variety of alternate cell lineages, likely because EBs develop regional differentiation over time in what appears to be a stochastic and spontaneous process. As a result, cultures that utilize an embryoid body step typically introduce an unpredictable degree of variability between differentiation attempts. To address this problem, several groups have either switched to a monolayer-style culture (often co-culture), and/or use hepatocyte reporter constructs that are only turned on at specific stages of development, or sort desired populations based on the expression of cell surface markers that are upregulated during the course of differentiation, or use three dimensional culture in addition to monolayer culture at specified times during the differentiation protocol (Basma et al., 2009; Cheng et al., 2012; Lavon, 2010; Ogawa et al., 2013; Takebe et al., 2013, 2014). Using these protocols, relatively pure subpopulations of cells can be isolated, and which have been tested functionally in a variety of hepatocyte transplantation assays. Unfortunately, the outcome of these experiments has varied widely, and to date, no published studies have demonstrated the ability to re-plate and use these cells in vitro (Basma et al., 2009). One creative solution involved a variety of these techniques including monolayer differentiation of pluripotent stem cells into hepatic specified endoderm and their combination with non-parenchymal cells (endothelial cells and mesenchymal cells) and Matrigel into self-organizing three dimensional gel structures which were then transplanted into immunodeficient mice (Takebe et al., 2013, 2014). Although in vitro function was similar to prior monolayer studies and did not demonstrate enhanced differentiated function as compared to primary adult human hepatocytes, these gel-incorporated cell clusters enabled transplantation, engraftment, vascularization and functional activity in vivo (Takebe et al., 2013). The engrafted gels enhanced the survival of the mice in a toxic injury model. Yet, despite the prediction that the in vivo environment would enable the terminal differentiation of iHLCs, the authors of this study did not observe complete maturation of iHLCs into adult hepatocytes in vivo (Takebe et al., 2013). An alternative approach is to identify factors that induce human iHLC differentiation. We used a small-molecule screening approach and identified several factors that can induce the maturation of human iHLCs, as well as expansion of human adult hepatocytes (Shan et al., 2013). Exposure to either of two of these small molecules yielded decreased fetal marker expression along with enhanced expression and functional activity of adult markers including cytochrome P450s (Shan et al., 2013). In a separate finding, Ogawa and colleagues showed that the addition of a cyclic AMP analog enhanced the differentiation state of iHLCs, based on decreased expression of fetal markers along with some elevation of adult marker expression (Ogawa et al., 2013). These studies represent continued optimization of iHLC differentiation protocols towards producing more mature cells.

2.2. Characterization of differentiated progeny

More recently, several groups have optimized the differentiation procedure and eliminated the use of poorly defined components such as serum, fibroblast feeder cells, embryoid bodies, and other undefined culture medium components, and have optimized their protocols based on a growing understanding of mouse hepatic development. These next generation protocols typically start with pluripotent stem cells and in a step-wise manner, expose the cultured cells to a series of defined factors in order to obtain iHLCs (Si-Tayeb et al., 2010; Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Touboul et al., 2010). According to these methods, iHLCs are obtained with a much higher efficiency than earlier aggregation protocols, although scalability remains a challenge in light of the numbers of cells that may be required for future applications. The key to the success of these protocols was the ability to promote the pluripotent cells to adopt a definitive endoderm fate at higher efficiency. First described in 2006 using embryonic stem cells, this class of protocols identified the role that activin A and WNT3 signaling play during the establishment of the early primitive streak, which ultimately leads to endoderm specification (D'Amour et al., 2005; Hay et al., 2008). These insights opened the door to further refinements in the methodology, and enabled the derivation of visceral, endodermal-derived tissues. These updated protocols yield hepatic lineage cells that can be considered hepatic-like based on morphologic and some phenotypic analyses (Si-Tayeb et al., 2010; Song et al., 2009; Touboul et al., 2010). However,

functional and other specific phenotypic analyses demonstrate that the resulting iHLCs exhibit an immature hepatic phenotype, in that they resemble fetal hepatocytes more than adult hepatocytes. Notably, iHLCs persistently express fetal markers like alpha-fetoprotein (AFP) and lack key mature hepatocyte functions, as reflected by drastically reduced activity (~0.1%) of many detoxification enzymes (e.g. CYP2A6, CYP3A4) (Si-Tayeb et al., 2010; Song et al., 2009; Touboul et al., 2010). These subtle but important differences between iHLCs and primary adult hepatocytes have limited the use of stem cells as a renewable source of functional liver cells.

Despite the development of multiple differentiation protocols, it is unclear as to which protocol leads to the production of the most terminally differentiated hepatocyte-like cells. This challenge in comparison with adult-derived cells typically stems from the inclusion of only minimally detailed functional analysis. Therefore, it is important to review the various approaches that numerous labs use to validate the identity of the lineages and precursors that result from hepatic differentiation methodologies. Indeed, in order to optimize a protocol designed to yield efficient, robust progeny of a desired lineage, it is essential that appropriate functional tests are performed, and that reasonable control and 'benchmark' comparisons are made as part of the refining process. As an example, we have summarized three differentiation protocols established by Si-Tayeb et al. (2010), Song et al. (2009), and Touboul et al. (2010). All protocols share a step-wise process to generate definitive endoderm, then hepatic-specified endoderm, followed by hepatoblasts, and finally yield hepatocytes, although the precise culture conditions, growth factor combinations, kinetics, efficiency, and overall protocol complexity vary in each case (Fig. 1). In terms of the reported characterization of the cells generated during the course of the procedures, Si-Tayeb et al. completed immunofluorescence analysis, periodic acid Schiff staining for glycogen production, LDL uptake, albumin secretion, and in vivo transplantation. Song et al. completed immunofluorescence analysis, periodic acid Schiff staining for glycogen production albumin production assay, urea metabolism assay, and CYP2B analysis. Touboul et al. completed immunofluorescence analysis, flow cytometry analysis, indocyanine green assay, CYP3A5/CYP3A7 (fetal cytochrome P450) analysis and in vivo transplantation. All three of these examples highlight that, particularly in the case of the hepatocyte lineage, no single assay can conclusively confirm a hepatic identity in isolation. However, as illustrated by these three publications, there are a multitude of morphologic, phenotypic, and functional tests that can be combined to demonstrate with confidence that iHLCs are similar in quality and functions to primary adult human hepatocytes. We describe a panel of these assays below.

2.3. Cellular morphology

As with their primary hepatocyte counterparts, iHLCs should be cuboidal or polygonal in appearance and have enlarged nuclei with an increased cytoplasm to nucleus ratio. In electron microscopy studies, abundant mitochondria, peroxisomes, lipid bodies, intact golgi apparatus, and rough endoplasmic reticulum should be present. In addition, abundant microvilli and vesicles should be visible, and junctional complexes should be present on either side of nearby hepatocytes, consistent with the presence of a bile canalicular network.

2.4. Gene and protein expression

In addition to the overall morphology of the population, confirming lineage identity depends on demonstrating the presence or absence of expression of particular genes and proteins. Based on developmental studies in vivo, numerous expected molecular phenotypes have been defined throughout the differentiation process as pluripotent cells transition to an adult hepatocyte state. Table 1 outlines expected expression patterns for various stages along the differentiation cascade. In terms of mRNA expression analysis, the preferred methodology is to use quantitative real-time PCR (qRT-PCR) analysis of iHLCs compared to a high-quality human hepatocyte reference (see below). At each differentiation step, it is important to characterize and demonstrate the commitment or specification to the specified lineage. Protein immunofluorescence or immunohistochemistry staining should be performed to confirm qRT-PCR data as well as to help determine marker co-expression. This per-cell assay also allows for an assessment of overall differentiation efficiency within a given culture well or plate. Cell polarization, an essential feature of functional hepatocytes, can also be examined in this manner, in that several proteins should only be expressed on apical (e.g. BSEP, MRP-2, P-gp) or basolateral (e.g. CD26, NTCP, OATP) membranes. Finally, at least a selection of the imaging stains should be confirmed by Western blot of bulk populations to decrease the possibility of off-target staining results.

2.5. Functional characterization

The iHLCs should exhibit functional characteristics of hepatocytes, in addition to their morphologic and phenotypic traits. Hepatocytes are notable for their wide variety of metabolic and other functional capacities, spanning over 500 classes of functions such as energy metabolism, bile production, and synthetic or detoxification functions, and thus to conclusively validate their identity, a series of in vitro assays can be performed to demonstrate activity in a variety of organized functional categories. Table 2 provides the most commonly used assays for hepatocytes and, in our experience, represent a good set to assess differentiated liver functions in both primary hepatocyte and iHLC cultures.

In vitro cell culture may enable phenotypic and functional iHLC characterization across a variety of individual parameters, however, a higher bar – and to some, a necessary bar to reach – is to achieve in vivo engraftment and function, and in some cases, functional rescue. Functional rescue of hepatic function implies that at least some functional requirements of the native liver, including protein secretion (i.e. albumin,

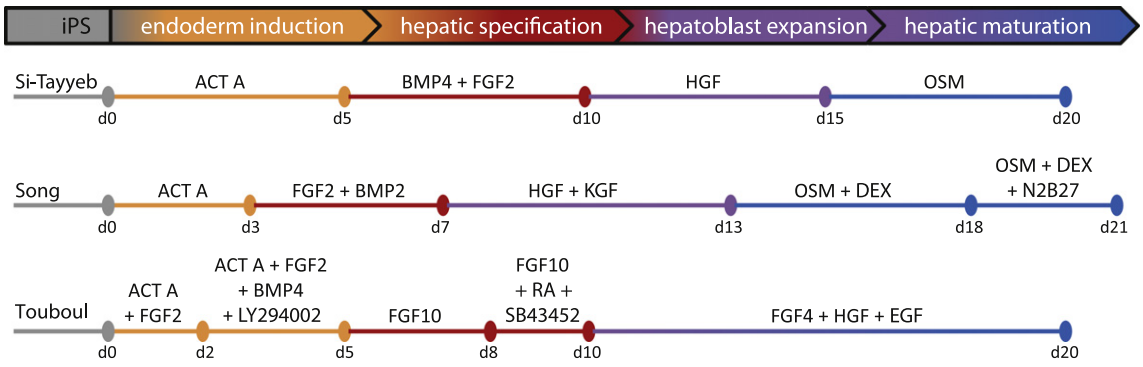

Fig. 1. Generation of hepatocyte-like cells from iPS cells via three stepwise protocols. Schematic outlining the differentiation kinetics and growth factors utilized in three different but commonly used protocols (Si-Tayeb et al., 2010; Song et al., 2009; Touboul et al., 2010).

Table 1
Marker expression throughout hepatocyte differentiation.

Gene	iPS	Definitive endoderm	Hepatic specified endoderm	Hepatoblast specification	Fetal hepatocyte	Adult hepatocyte
OCT3/4	+	−	−	NT	NT	very low
Nanog	+	−	−	NT	NT	−
SSEA4	+	−	−	NT	NT	−
TRA1-60	+	−	−	NT	NT	−
FOXA2	−	+	+, lower	+	+	+
GATA-4	−	+	+, lower	+	+	+
Cerberus	−	+	−	−	−	−
FGF17	−	+	−	−	−	−
Goosecoid	−	+	−	−	−	−
HNF4α	−	−[a]	+	+	+	+
hHex	−	+	+, very low	−	−	−
MixL1	−	+	−	−	−	−
Sox7	−	−[a]	−	−	−	−
Sox17	−	+	+, lower	+, lower	+, lower	+, very low
APOA1	−	−	+	+	+	+
APOB	−	−	+	+	+	+
BMP6	−	−	+	−	−	−
DUSP6	−	−	+	−	−	−
TBX3	−	−	+	+	+	+
AFP	−	−	−	+	+	−
Decorin	−	−	−	+	+	+
HNF1α	−	−	−	+	+	+
GSTA1	−	−	−	+	+	+
KRT19	−	−	−	+	+/−	−
TTR	−	−	−	+	+	+
α1AT	−	−	−	−	+	+
Albumin	−	−	−	+/−	+	+
ASGPR1	−	−	−	−	−	+
CPS1	−	−	−	−	−	+
CK8	−	−	−	−	+	+
CK18	−	−	−	−	+	+
CYP1A2	−	−	−	−	−	+
CYP2A6	−	−	−	−	−	+
CYP2B6	−	−	−	−	−	+
CYP2C9	−	−	−	−	−	+
CYP2C19	−	−	−	−	−	+
CYP2D6	−	−	−	−	−	+
CYP3A4	−	−	−	−	−	+
CYP3A7	−	−	−	−	+	Very low
MAOA/B	−	−	−	−	Very low	+
UGT1A1	−	−	−	−	−	+
MRP2	−	−	−	−	Very low	+
BSEP	−	−	−	−	Very low	+

[a] Expression of Sox7 or HNF4α at the definitive endoderm stage indicates production of embryonic rather than definitive endoderm.

alpha-1-antitryrpsin, coagulation factors), lipid metabolism, protein catabolism and urea production, drug detoxification, and bile production, are replaced by the engrafted cells. In the case of functional human HLC replacement, one would expect the humanization process to yield a host animal that harbors measurable human protein products, bile acids, lipid products, drug metabolism, drug toxicity, and drug metabolite formation. In conducting these assays, there are three essential sets of decisions that must be considered: choice of recipient model and route/dose of administered cells, evaluation methods used to determine the kinetics and extent of donor cell engraftment, and specific functional outcomes examined to assess both local and systemic functional output of the engrafted cells. A variety of models are available, each with relative strengths and weaknesses. Two immunodeficient, metabolic mutation mouse models that have often been utilized to assay for robust engraftment and repopulation of primary human hepatocytes include $Fah^{-/-}/Rag2^{-/-}/IL2r\gamma^{-/-}$ or alb-uPA severe combined immunodeficient mice (Azuma et al., 2007; Dandri et al., 2001). These models offer benefits in that endogenous liver cells are compromised, and thus minimal donor cell engraftment may lead to a measureable functional read out, and yet – as with most animal models – there remain some questions as to the absolute relevance of the outcome, and whether engraftment in this setting will directly correlate with function in a human. Nonetheless, although no iHLC transplant studies to date have observed significant repopulation with functional activity, small islands of stem cell derived hepatocyte-like cells have been detected using these compromised hosts. In these studies minimal to no function was detected (i.e. human albumin or alpha-1-antrypsin secretion in the mouse serum).

Highlighting the importance of selecting an appropriate host model for functional engraftment studies, a recent report described very high levels of albumin staining following the transplantation of iHLCs in a mouse model that has been traditionally utilized for toxicity testing of candidate drugs (Liu et al., 2011). In this case, the stem cell-derived donor cells did not carry any genetic or functional advantage over the surviving host cells, but did appear to be selected to repopulate the damaged liver. Unfortunately, the degree to which the local human albumin-staining cells contributed to circulating human markers of functional hepatocyte integration was extremely and surprisingly low. Overall, the unusually high levels of engraftment are encouraging for the field, but clearly additional work remains to be done to demonstrate fully functional engraftment of iHLCs in an in vivo setting. Given these observations, some researchers have begun to explore alternate animal models, including rodents. Indeed, one group has presented data in oral presentations showing that radiation preconditioning of host rats can enhance the engraftment of human hepatocytes and iHLCs. Consequently, it seems likely that identifying the most amenable, but appropriate, host model is both a central challenge and an active area of investigation in this field.

Table 2
Liver functions to measure in iHLCs and compare to levels in primary human hepatocytes. Abbreviations: LDL (low density lipoprotein), UGT (UDP-Glucuronosyl Transferase), SULT (sulfotransferase).

Category	Relevant assays
Synthetic function	• Albumin production (Schwartz et al., 2005) • Alpha-fetoprotein production (Chiao et al., 2008) • Alpha-1-antitrypsin production (Chen et al., 2011)
Energy metabolism	Lipid metabolism • LDL-R expression (Cayo et al., 2012) • LDL uptake (Cayo et al., 2012) Glucose metabolism • Gluconeogenesis (Khuu et al., 2011) • Glycogen production (Si-Tayeb et al., 2010) • Glucose-6-Phosphatase activity (Khuu et al., 2011) Protein metabolism • Ammonia uptake/urea production (Chen et al., 2011)
Bile production and metabolism	• Production and secretion of bilirubin monoglucuronide and diglucuronide (Tada et al., 1998) • Uptake of bile acids and secretion into hepatic biliary canalicular networks (Murray et al., 2011) • 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate secretion into canaliculi (Khetani and Bhatia, 2008)
Detoxification	• CYP3A4 expression • CYP3A7 expression Phase I–II enzyme activities (enzyme substrate: metabolite to measure) • CYP1A2 (phenacetin: acetaminophen) (Pillai et al., 2013) • CYP2A6 (coumarin: 7-hydroxycoumarin) (Khetani and Bhatia, 2008) • CYP2B6 (bupropion: hydroxybupropion) (Khetani and Bhatia, 2008) • CYP2C8 (paclitaxel: 6α-hydroxy-paclitaxel) (Vaclavikova et al., 2004) • CYP2C9 (tolbutamide or CYP2C9-Glo: 4-hydroxy-tolbutamide or luciferin) (Prot et al., 2011) • CYP2C19 (S-mephenytoin: 4-hydroxy-S-Mephenytoin) (Pillai et al., 2013) • CYP2D6 (dextromethorphan: dextrorphan) (Prot et al., 2011) • CYP2E1 (chlorzoxazone: 6-hydroxy-chlorzoxazone) (Ubeaud et al., 2001) • CYP3A4 (testosterone or CYP3A4-Glo: 6β-hydroxy-testosterone or luciferin) (Khetani and Bhatia, 2008) • UGT (7-hydroxycoumarin: 7-hydroxycoumarin-glucuronide) (Khetani and Bhatia, 2008) • SULT (7-hydroxycoumarin: 7-hydroxycoumarin-sulfate) (Khetani and Bhatia, 2008)

2.6. Selecting a 'gold-standard'

Finally, in all of the assays utilized to characterize candidate iHLC populations, particularly their functional traits, comparison with an appropriate reference cell type is critical. Hepatocyte reference controls such as cell lines or immortalized hepatocytes have been used extensively, and offer a reproducible benchmark population, however, these lines do not exhibit appropriate, physiological levels of most hepatocyte-specific functions(Gerets et al., 2012; Wilkening et al., 2003). Freshly isolated or uncultured, cryopreserved primary human hepatocytes are generally considered the ideal reference controls for this purpose. However, many functional tests require that cells are cultured for periods of hours to days, and once maintained in traditional culture systems, the morphology, phenotype, and functions of primary human hepatocytes all decline rapidly. Several updated in vitro platforms have been developed to preserve hepatic morphology, phenotype, and many functions of primary adult hepatocytes (i.e. Matrigel® overlay, collagen gel sandwich, micropatterned cocultured hepatocytes, and three-dimensional aggregates) (Dunn et al., 1989; Khetani and Bhatia, 2008; LeCluyse et al., 1994; Wu et al., 1996, 1999). One of these culture systems, discussed in more detail below, aligns primary human hepatocytes on a defined micropattern of ECM in coculture with supportive murine embryonic fibroblasts to control both homotypic and heterotypic interactions (micropatterned co-cultures or MPCCs). The MPCC platform has been shown to faithfully recapitulate hepatic morphology and numerous liver functions for 4–6 weeks in vitro (Khetani and Bhatia, 2008). Other alternatives include human liver tissue homogenates, human liver microsome isolation, and tissue sections that have been shown to faithfully recapitulate gene expression, cytochrome P450 activity and hepatocyte phenotype, although these systems cannot be used for dynamic studies. In some cases, characterization assays that do not require periods of culture, such as gene expression, one of the aforementioned alternatives or uncultured primary human hepatocytes offer a sufficient 'benchmark'. However, for other methods that require sample collection or observation over time in culture, we encourage the use of platforms such as MPCCs (or similarly highly functional and long-lasting platforms) for benchmarking purposes.

Unfortunately, while dozens of published papers demonstrate HLC production from a variety of cell types, including pluripotent stem cells, the criteria used to identify the resulting hepatocyte-like cells have varied from report to report. Due to this lack of standardization of what defines an HLC, it has been difficult to compare the relative success of various protocols, and thus it is close to impossible to identify candidate method modifications that may enhance iHLC production. As mentioned above, many groups have benchmarked their iHLC populations against cultured primary human hepatocytes, which contribute to the challenge in comparing results across platforms due to the variability in most human hepatocyte culture systems. Furthermore, multiple publications have established that unless specific culture model systems are employed, such as the MPCC system, cultured hepatocytes rapidly lose their phenotype and the majority of typical functions (LeCluyse, 2001). Moreover, cultured human hepatocytes upregulate inappropriate and often immature markers such as AFP. Consequently, any comparisons made to these altered and declining adult hepatocytes may make the candidate iHLCs appear more functionally mature than is the case in vivo. Indeed, examination of published accounts reveals that many protocols lead to fetal hepatocyte-like cells, although in some cases the characterization reported is not sufficient to determine the fetal versus mature nature of the resulting HLCs. Given the seemingly fetal nature of iHLCs produced to date, it is apparent that additional, careful modification of differentiation protocols will be required before the potential of these cells can be realized.

2.7. Additional practical considerations

Even after reaching a suitably characterized iHLC state, a remaining issue is the question of the stability of their phenotype and function in long-term culture, similar to the alterations observed in cultured adult hepatocytes (Guillouzo, 1998). Consequently a variety of strategies have been undertaken to improve the hepatocyte-specific functions and survival of primary hepatocytes in vitro. Numerous studies have focused on strategies that aim to recapitulate the normal liver microenvironment and provide missing microenvironmental cues including soluble factors (Guillouzo, 1998; Jindal et al., 2009), cell–matrix interactions (Bissell et al., 1987; Flaim et al., 2005; Lin et al., 2004), and heterotypic cell–cell interactions with non-parenchymal cells (Bhatia et al., 1999; March et al., 2009). Microfabrication approaches (semiconductor-driven microtechnology tools which enable micrometer-scale control over cell adhesion, shape and multi-cellular interactions) have been utilized to control tissue microarchitecture in order to define the best cell–ECM interactions and to achieve an optimal balance of homotypic and heterotypic cellular interactions to promote hepatocyte function (Folch and Toner, 2000; Fukuda et al., 2006). These approaches have culminated in photolithographic cell patterning techniques and robust hepatocyte culture model systems, such as the aforementioned MPCC platform, which has been used extensively for drug development and pathogen modeling (Chan et al., 2013; Khetani et al., 2013; March et al., 2013; Ploss et al., 2010; Wang et al., 2010). Utilization of these or related platforms will be critical to improving the development and culture of iHLCs and will not only improve the robustness of the iHLC system, but will also help untangle the role that soluble factors, cell–ECM interactions and homotypic and heterotypic cell–cell interactions play in hepatic development. Notably, this area of study has also raised the possibility that the observed heterogeneity of cell types produced using current iHLC differentiation protocols may actually be required for robust iHLC differentiation. That is, during the course of iHLC generation, differentiating progenitors may require signals produced by other cell types developed in parallel. Thus, it may not be possible to achieve a pure population of functional iHLCs, unless replacement signals and/or factors can be defined and provided with appropriate kinetics. All of the above underscore the need for live cell reporters that allow differentiation state to be monitored, and permit subsequent isolation of desired populations.

The differentiation protocols currently used to coax pluripotent stem cells to generate hepatocyte-like cells rely on the addition of exogenous growth factors identified in developmental studies. Subsequent work has established the key transcription factors that are activated during the course of the hepatic differentiation process. Other groups have demonstrated that direct reprogramming strategies which bypass the pluripotent stage can be applied to convert fibroblasts into cardiomyocytes and neurons by overexpressing key lineage-specific transcriptional regulators. Motivated by these findings, two groups have recently shown that mouse fibroblasts can be reprogrammed into hepatocyte-like cells via the overexpression of key transcription factors (i.e. Foxa2/3, HNF1α and GATA4 or Foxa1/2/3 and HNF4α) (Huang et al., 2011; Sekiya and Suzuki, 2011). In both examples, induced hepatocytes exhibited only minimal hepatocyte-specific functions including very low albumin secretion, triglyceride synthesis, and cytochrome P450 function. Transplantation of induced hepatocytes into FAH deficient mice (a genetic model of hereditary tyrosinemia that develops liver failure without NTBC drug treatment) led to liver repopulation and survival of less than half of the hosts. This partial success stands in contrast to reported outcomes using primary mouse hepatocytes which repopulate the complete liver with rare failure to rescue the mice from death. Whether these differences in repopulation and in vitro hepatic function reflect variable reprogramming, deregulated function or immature cells is still unclear.

2.8. Potential in vitro applications of iHLCs

iPS and iHLCs offer a dizzying array of opportunities including hepatocyte-like cell generation for possible cell replacement therapy. However, this therapeutic goal is likely still far on the horizon, and thus their greatest strength, or at least the most near-term potential of iHLCs, may lie in applying them to serve as a platform for disease modeling, or for mechanistic toxicity studies in idiosyncratic responses. The cost of drug development is heavily influenced by the attrition rate of tested compounds; for every drug that reaches the marketplace, 5000 to 10,000 molecules are tested in a preclinical setting (Kola and Landis, 2004). Utilizing iHLCs for this sort of application will require an experimental platform that is robust and scalable, and can be applied to important evaluations of drug disposition and toxicity (Table 3). Not only can iHLCs provide an unlimited supply of liver cells for drug testing, but also the ability to generate iHLCs from different donors can provide an assessment of donor-specific drug responses in vitro. However, generation of iHLCs from specific patients with specific genotype or diseases will involve multiple essential steps. First, a target disease (or polymorphism) that exhibits a recognizable in vitro phenotype must be selected, and candidate tissue donors with the target disease need to be identified, as well as healthy control subjects. Second, iPS cells need to be derived, characterized, and grown in an easily scalable platform. Third, differentiation of iPS cells into iHLCs needs to be efficient, complete, consistent, and conducted in a format appropriate for large-scale small molecule testing (i.e. at least 96, 384, or 1024 wells). While, as discussed above, current best-available iHLCs remain incompletely differentiated, reports of the successful application of this process have been described for patients with alpha-1-antitrypsin disease and familial hyperlipidemia (Cayo et al., 2012; Rashid et al., 2010). However, in the case of polygenic, and thus genetically complex diseases such as nonalcoholic fatty liver disease, screening for a specific phenotypic or functional outcome may not be possible, even after new methods have been developed to achieve more complete and robust stem cell-derived hepatocyte production. Consequently, no matter what disease is being interrogated, a differentiation platform designed for screening or modeling must produce pure populations of fully differentiated cells with minimal heterogeneity and with no stochasticity. Certainly, further improvements currently under examination by the stem cell and engineering communities such as efforts to identify microenvironmental signals to increase the purity, efficiency and maturation of desired cell types are likely to help tackle and solve these various problems.

iHLCs can also enable studies of pathogens that exclusively target human hepatocytes, notably those with profound global health implications such as hepatitis B virus (HBV), hepatitis C virus (HCV) and malaria. For example, current HCV model systems utilize the Huh7 hepatoma carcinoma cell line to examine the HCV viral life cycle in vitro which is limited by the poor hepatic function of the line (Lindenbach et al., 2005; Wakita et al., 2005). Consequently, recent approaches using micropatterning techniques have enabled HCV infection in primary human hepatocytes (Ploss et al., 2010). More recently, we demonstrated that iHLCs express all known entry factors of HCV, support the complete HCV viral cycle, and exhibit a robust anti-viral immune response (Schwartz et al., 2012). Wu et al. then showed that permissiveness for HCV infection was differentiation-stage dependent upon the expression of the microRNA-122 (Wu et al., 2012). iHLCs can thus serve as a platform to study defined stages of pathogen permissiveness, explore the role that host genetics plays in pathogenesis and elucidate the role that these host factors play in disease pathogenesis.

3. Conclusions

In summary, pluripotent stem cell derived hepatocyte-like cells can be generated from iPS cells and hESC in a reproducible and efficient

Table 3
Hepatocyte assays relevant for evaluating drug disposition and toxicity. Abbreviations: LC–MS/MS (liquid chromatography–mass spectrometry), LDH (lactate dehydrogenase), AST (aspartate transaminase), ALT (alanine aminotransferase), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), GSH (glutathione), MMP (mitochondrial membrane potential), Cyt-C (cytochrome C), and ROS (reactive oxygen species).

Category	Relevant assays	Prototypical test compounds
Drug clearance	• Measurement of drug concentration in culture supernatants over hours to days (depending on expected turnover) using LC–MS/MS	• Verapamil (high turnover) (Chao et al., 2009) • Diclofenac (medium turnover) (Chan et al., 2013) • Warfarin (low turnover) (Chan et al., 2013)
Metabolite identification	• Identification of 1 or more drug metabolites present in culture supernatants over hours (primary metabolites) to days (secondary metabolites) using LC–MS/MS • Quantitation of metabolites via radiolabeled drugs	• Ziprasidone Phase I primary and secondary metabolites (ziprasidone sulfoxide, *S*-methyldihydroziprasidone) (Wang et al., 2010) • Gemcabene Phase II primary metabolite (gemcabene-glucuronide) (Wang et al., 2010) • Traxoprodil Phase II secondary metabolite (traxoprodil-methoxy-sulfate) (Wang et al., 2010)
Drug–drug interactions	• Measurement of enzyme activity using prototypical substrates after incubation with inducer drugs (days) • Measurement of enzyme activity using prototypical substrates after incubation with competitive (hours) or time-dependent inhibitor drugs (days)	*Phase I–II enzyme (inducer, inhibitor)* (Khetani and Bhatia, 2008; Madan et al., 2003; USDHHS et al., 2008) • CYP1A2 (omeprazole, ciprofloxacin) • CYP2B6 (phenobarbital, clopidogrel) • CYP2C8 (rifampin, gemfibrozil) • CYP2C9 (rifampin, fluconazole) • CYP2C19 (rifampin, ticlopidine) • CYP3A4 (rifampin, itraconazole) • UGT (probenecid)
Drug toxicity	• Measurement of markers in culture supernatants (i.e. albumin, urea, LDH, AST, ALT) (Khetani et al., 2013) • Measurement of markers in cell lysates (i.e. MTT, ATP, GSH) (Khetani and Bhatia, 2008; Khetani et al., 2013) • High content fluorescent imaging of organelle dysfunction (i.e. dyes for MMP, GSH, Cyt-C, nuclei, ROS). Can be automated with proper equipment (Xu et al., 2008)	*Toxic/non-toxic pairs:* • Tolcapone/entacapone (Khetani et al., 2013) • Troglitazone/rosiglitazone (Khetani and Bhatia, 2008) • Ibufenac/ibuprofen • Trovafloxacin/levofloxacin (Khetani et al., 2013) • Alpidem/zolpidem *Other prototypical toxic drugs:* • Amiodarone (Khetani et al., 2013) • Ketoconazole (Khetani et al., 2013)

manner. Review of several available methods reveals that there are multiple paths that lead from pluripotency to at least an immature hepatic phenotype that more closely resembles fetal rather than adult hepatocytes. This apparent incomplete differentiation state likely results from our poor understanding of the mechanisms underlying the developmental shift from fetal to adult liver. Moreover, the existing lack of standardization of morphologic, phenotypic, and functional characterization of iHLCs has made comparisons between published papers challenging, if not impossible. In this review, we have illustrated the importance of extensive phenotypic and functional characterization and we encourage the community to apply various standards during hepatocyte-like cell characterization. In addition, the use of well-documented and functional hepatocyte reference controls is key to the future improvement of iHLC generation. This advance will lead to the rapid adoption of this key population and their use in a variety of applications including the study of the mechanisms of human disease and development, and, perhaps in the longer term, as a platform for cell based therapeutics and to evaluate the efficacy and toxicity of pharmaceuticals.

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